

AA



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07H 21/00, A61K 31/70</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 95/22554</b> <b>(43) International Publication Date:</b> 24 August 1995 (24.08.95)
<b>(21) International Application Number:</b> PCT/US95/02082 <b>(22) International Filing Date:</b> 17 February 1995 (17.02.95) <b>(30) Priority Data:</b> 08/199,510                      18 February 1994 (18.02.94)                      US <b>(71) Applicant:</b> HYBRIDON, INC. [US/US]; One Innovation Drive, Worcester, MA 01605 (US). <b>(72) Inventor:</b> MULDER, Carel; 10 Germain Street, Worcester, MA 01602-2105 (US). <b>(74) Agents:</b> KERNER, Ann-Louise et al.; Lappin & Kusmer, 200 State Street, Boston, MA 02109 (US).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CN, CZ, DE, DK, EE, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> OLIGONUCLEOTIDES WITH ANTI-EPSTEIN-BARR VIRUS ACTIVITY  <b>(57) Abstract</b>  Disclosed are oligonucleotides complementary to and hybridizable with a portion of the BZLF1 RNA of Epstein-Barr virus, useful for inhibiting the induction of the lytic cycle in EBV-infected cells, and in inhibiting EBV replication.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

-1-

OLIGONUCLEOTIDES WITH  
ANTI-EPSTEIN-BARR VIRUS ACTIVITY

BACKGROUND OF THE INVENTION

This invention relates to the treatment of Epstein-Barr virus (EBV) infection. More particularly, this invention relates to antisense oligonucleotides complementary to portions of the viral mRNA useful in inhibiting Epstein-Barr virus replication.

Epstein-Barr virus (EBV) is a human herpesvirus having a double-stranded DNA genome. It is a B lymphotropic virus which is worldwide in distribution. Primary infection with EBV during childhood is usually subclinical. Between 25 and 70 percent of adolescents and adults who undergo a primary EBV infection develop the clinical syndrome of infectious mononucleosis. EBV is also associated with EBV syndrome, X-linked lymphoproliferative syndrome (XLP), and oral leucoplacia in AIDS patients, and with malignancies such as nasopharyngeal carcinoma, Burkitt's lymphoma, anaplastic nasopharyngeal carcinoma, Hodgkin's Disease, and hairy cell leukemia.

The diseases which have been attributed to EBV infection are diverse in part because, after entering the body via the oropharynx and entering an epithelial cell, the viral genome can replicate in alternative ways, leading to productive (lytic) or latent infection. EBV lytic infection causes

-2-

the pathology related to the cytololysis of infected cells, often accompanied by the body's abberant immunopathologic responses to viral antigens. In contrast, EBV latent infection occurs when the viral genome becomes fixed in epithelial or lymphoid cells as an episome, rather than in a viral particle. Episomes later appear in anaplastic nasopharyngeal carcinoma and in immortalized B-lymphocytes.

Productive EBV infection such as that associated with X-linked lymphoproliferative syndrome has been treated systematically with conventional anti-virus nucleoside analogs such as 9-(2-hydroxyethoxymethyl)guanine (acyclovir), 1-(2-fluoro-2-deoxy-B-D-arabinofuranosyl)-5-iodocytosine (FIAC), several E-5-(2-bromovinyl)-2'-deoxyuridine (BVdU)-containing compounds, and (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine ((S)-HPMPA) which traditionally have been used to inhibit the replication of other herpesviruses, as well as azido-thymidine (AZT) (reviewed in Pagano (in press). Pyrophosphate analogs, thymidine kinase analogs, and ribonucleoside reductase inhibitors have also been used. However, none of these drugs have been very effective in inhibiting EBV replication. In addition, the effect of these analogs is not limited to viral nucleotides, and thus unwanted side effects often present when they are used. Other therapies for EBV-associated disorders such as nasopharyngeal carcinoma have included surgery in combination with high doses of radiation. Radiation therapy has also been combined with

-3-

chemotherapy in the treatment of EBV-related lymphomas, but often without much success. Thus, there remains a need for more effective treatment of EBV-related diseases which are specific for the virus, and which have few, if any, detrimental side-effects.

Recently, new chemotherapeutic agents have been developed which are capable of modulating cellular and foreign gene expression. These agents, called antisense oligonucleotides, bind to a target single-stranded nucleic acid molecules according to the Watson-Crick or the Hoogsteen rule of base pairing, and in doing so, disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal transcription, splicing, or translation; by triggering the enzymatic destruction of mRNA by RNase H, or by destroying the target via reactive groups attached directly to the antisense oligonucleotide.

Antisense oligodeoxynucleotides have been designed to specifically treat latent EBV infection by inhibiting the expression of the EBNA-1 gene of EBV (U.S. Patent No. 5,242,906). Antisense oligonucleotides have also been used to inhibit the expression of HIV-1, influenza, and other viruses (see, e.g., Agrawal et al., U.S. Patent No. 5,194,428; Pederson et al., U.S. Patent Nos. 5,149,797; Agrawal (1992) *Trends Biotechnol.* 10:152-158; Agrawal et al. in *Gene Regulation: Biology of Antisense RNA and DNA* (Erickson and Izant, eds.) Raven Press Ltd., New York (1992) pp. 273-283);

-4-

Matsukura et al. in *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS*, Wiley-Liss, Inc. (1992) pp. 159-178; and Agrawal (1991) in *Prospects for Antisense Nucleic Acid Therapy for Cancer and AIDS*, (Wickstrom, ed.)  
5 Liss, New York, pp. 145-148).

A need for new strategies still remains for the treatment and prevention of EBV infections. In particular, compositions and therapeutic  
10 methods utilizing these compositions are desired which are effective and whose use are accompanied by little or no cellular toxicity.

#### SUMMARY OF THE INVENTION

15 Infection by EBV may result in latent or lytic infection. The activation of the lytic cycle has been found to involve several trans-activating EBV genes, BZLF1, BRLF1, and BMLF1  
20 (Chevallier-Greco et al. (1986) *EMBO J.* 5:3243-3249; Hardwick et al. (1988) *J. Virol.* 62:2274-2284; Lieberman et al. (1986) *J. Virol.* 60:140-148; Takada et al. (1986) *J. Virol.* 57:1016-1022). However, only the BZLF1 gene, the first "immediate early" gene,  
25 by itself, is able to induce the lytic cycle in latently infected B lymphocytes, (Chevallier-Greco et al. (1986) *EMBO J.* 5:3243-3249; Countryman et al. (1985) *Proc. Natl. Acad. Sci. (USA)* 82:4085-4089; Takada et al. (1986) *J. Virol.* 57:1016-  
30 1022), indicating that the initial event in the lytic cycle is activation of BZLF1 transcription.

-5-

The present invention includes methods of suppressing the induction of the lytic (replicative) cycle of EBV using synthetic oligonucleotides complementary to and hybridizable with BZLF1 RNA.

As used herein, the term "synthetic oligonucleotide" includes chemically synthesized polymers of three or up to 100 and preferably from about 12 to about 60 ribonucleotide and/or deoxyribonucleotide monomers connected together or linked by at least one 5' to 3' internucleotide linkage.

In some aspects of the invention, the oligonucleotides are modified. The term "modified oligonucleotide" is used herein as an oligonucleotide in which at least two of its nucleotides are covalently linked via a synthetic linkage, i.e., a linkage other than a phosphodiester between the 5' end of one nucleotide and the 3' end of another nucleotide in which the 5' nucleotide phosphate has been replaced with any number of chemical groups. Preferable synthetic linkages include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphate esters, carbamates, carbonates, phosphate triesters, acetamidate, and carboxymethyl esters. In one preferred embodiment of the invention, the oligonucleotide comprises at least one phosphorothioate linkage.

-6-

The term "modified oligonucleotide" also encompasses oligonucleotides with a modified base and/or sugar. For example, a 3', 5'-substituted oligonucleotide is a modified oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position). A modified oligonucleotide may also be a capped species. In addition, unoxidized or partially oxidized oligonucleotides having a substitution in one nonbridging oxygen per nucleotide in the molecule are also considered to be modified oligonucleotides. Also considered as modified oligonucleotides are oligonucleotides having nuclease resistance-conferring bulky substituents at their 3' and/or 5' end(s) and/or various other structural modifications not found *in vivo* without human intervention are also considered herein as modified.

The oligonucleotides of the invention are complementary to a portion of the EBV RNA transcribed from the immediate early BZLF1 gene. The oligonucleotides hybridize to the RNA under normal physiological conditions existing within a cell harboring the RNA. Such conditions include pH, temperature, and ionic conditions.

The portion of the BZLF1 RNA to which the oligonucleotides of the invention are complementary includes any region of the RNA. This region is at least 6, and preferably 12 to 60 nucleotides in length. In most preferred aspects



-7-

of the invention, the oligonucleotides are 15 to 25 nucleotides in length. In some particular embodiments, the region includes (starting from the 5' end of the mRNA) nucleotides 1-25 (flanking the initiation codon (AUG), and having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1. In other embodiments, the oligonucleotide of the invention has a sequence which is complementary to the region starting with the 5' untranslated region and ending with the two copies of AUG with which the open reading frame begins, and having nucleotide SEQ ID NO:2. In yet another embodiment, the oligonucleotide has a sequence complementary to a region immediately downstream of the translational initiation codon of the mRNA derived from the BZLF1 gene, and has SEQ ID NO:3.

In some aspects, the invention provides an oligonucleotide which has antiviral activity against EBV lytic replication effected by hybridization with a portion of the mRNA. In others, a pharmaceutical composition including at least one of the oligonucleotides of the invention, and a pharmaceutically acceptable carrier are provided.

The pharmaceutical composition is used in a method of inhibiting, preventing, and/or reducing EBV replication in a cell. In this method, a therapeutic amount of the pharmaceutical composition is administered to the cell which is to be protected from lytic infection or treated for an existing infection. The oligonucleotide in

-8-

the pharmaceutical composition enters the cell,  
wherein it hybridizes to EBV DNA or RNA, thereby  
inhibiting EBV replication and thus lytic  
infection. The pharmaceutical composition is also  
5 utilized in a method of treating EBV infection  
wherein the composition is administered to an  
infected mammal or cell.

-9-

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1 is a graphic representation of the effect that no oligonucleotide treatment (A), a modified antisense oligonucleotide of the invention (B), a modified control oligonucleotide (C), a phosphodiester (PO)-linked (unmodified) oligonucleotide of the invention (D), and an unmodified control oligonucleotide (E) has on the expression of the EA<sub>p</sub> gene in EBV-infected cells;

FIG. 2A is a graphic representation of the induction of EA<sub>p</sub> in EBV-infected cells treated with different concentrations of unmodified control (-o-) or unmodified antisense (-●-) oligonucleotides;

FIG. 2B is a graphic representation of the induction of EA<sub>p</sub> in EBV-infected cells treated with different concentrations of modified control (-o-) or modified antisense (-●-) oligonucleotides;

FIG. 3A is a graphic representation of the induction of VCA in EBV-infected cells treated with different concentrations of modified control (-o-) or modified antisense (-●-) oligonucleotides;

-10-

FIG. 3B is a graphic representation of the induction of VCA in EBV-infected cells treated with different concentration of unmodified control (-o-) or unmodified antisense (-●-) oligonucleotides; and

FIG. 4 is a autoradiogram showing the effect on EBV DNA synthesis of adding 100  $\mu\text{g/ml}$  modified antisense oligonucleotides to Akata cells at different times after activation of the lytic cycle by IgG: lane 1, induced cells, no oligonucleotides; lane 2, uninduced cells; lane 3, antisense oligonucleotides added 3 hours before IgG activation of the lytic cycle; lane 4, antisense oligonucleotides added at the same time as IgG activation; lane 5, antisense added 3 hours after IgG activation. The numbers under each lane are the fractional amounts of counts in the linear (L) EBV DNA range compared with the counts in lane 1; and

FIG. 5A is an autoradiogram showing effect of 100  $\mu\text{g/ml}$  modified antisense or control oligonucleotides on EBV DNA synthesis of P3HR-1 cells at day 0 which were then subcultured every 2 days in medium containing 100  $\mu\text{g/ml}$  oligonucleotide: lanes 1, no oligonucleotide treatment; lanes 2, modified antisense oligonucleotide; lanes 3, modified control oligonucleotide. The numbers under each lane are the fractional amounts of counts in the linear (L) EBV DNA range compared with the counts in lanes 1; and

-11-

FIG. 5B is an autoradiogram showing effect of 200  $\mu$ g/ml modified antisense or control oligonucleotides on EBV DNA synthesis in P3HR-1 cells at day 0 which were then subcultured every 2 days in medium containing 200  $\mu$ g/ml oligonucleotide: lanes 1, no oligonucleotide treatment; lanes 2, modified antisense oligonucleotide; lanes 3, modified control oligonucleotide. The numbers under each lane are the fractional amounts of counts in the linear (L) EBV DNA range compared with the counts in lanes 1.

-12-

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patent and allowed applications cited herein are hereby incorporated by reference.

10 EBV has a double stranded DNA genome. During latency, this genome is circular; in the virion, and therefore during productive (lytic) infection, it is linear. BZLF1 is the first immediate early (IE) gene of EBV. Its translation product, ZEBRA, transactivates other IE genes whose products  
15 transactivate early (EA) genes. Most EA gene products are required for viral DNA synthesis. Once viral DNA synthesis starts, a third set of lytic viral genes are activated, the late (LA) genes, whose products are mainly viral particle  
20 (virion) proteins such as viral capsid antigen (VCA), the major capsid protein.

Activation of this entire lytic cycle can be prevented by the suppression of BZLF1 expression.  
25 Accordingly, the present invention relates to methods of suppressing BZLF1 expression by means of oligonucleotides which are sufficiently complementary to regions of the EBV BZLF1 RNA such that, under normal physiological conditions  
30 existing in the cell, they hybridize to those regions. Hybridization renders the mRNA unavailable to serve as a template for translation of protein, and as a result, EBV lytic infection and replication is inhibited.

-13-

The oligonucleotides may be targeted to any region of the BZLF1 transcript. An example of such oligonucleotide includes one complementary to the region including (starting from the 5' end of the mRNA) nucleotides 1-25 flanking the initiation codon AUG and having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1. Another example is an oligonucleotide having a sequence complementary to the region starting with the 5' untranslated region and ending with the two copies of AUG with which the open reading frame begins. Such an oligonucleotide can have the nucleotide sequence set forth in the Sequence listing as SEQ ID NO:2. Yet another example is an oligonucleotide having a sequence complementary to a region immediately downstream of the translational initiation codon of the mRNA derived from the BZLF1 gene. An oligonucleotide sequence fitting this description is set forth in SEQ ID NO:3.

Oligonucleotides of the invention are composed of deoxyribonucleotides, ribonucleotides, or a combination of both, with the 5' end of one nucleotide and the 3' end of another nucleotide being covalently linked. These oligonucleotides are at least 6 nucleotides in length, but are preferably 10 to 50 nucleotides long, with 15 to 25mers being the most common. These oligonucleotides can be prepared by the art recognized methods such as phosphoramidate or H-phosphonate chemistry which can be carried out manually or by an automated synthesizer as

-14-

described in Uhlmann et al. (*Chem. Rev.* (1990)  
90:534-583).

5       The oligonucleotides of the invention may  
also be modified in a number of ways without  
compromising their ability to hybridize to BZLF1  
mRNA. For example, the oligonucleotides may  
contains other than phosphodiester internucleotide  
10       linkages between the 5' end of one nucleotide and  
the 3' end of another nucleotide in which the 5'  
nucleotide phosphate has been replaced with any  
number of chemical groups. Examples of such  
chemical groups include alkylphosphonates,  
phosphorothioates, phosphorodithioates,  
15       alkylphosphonothioates, phosphoramidates,  
phosphate esters, carbamates, acetamidate,  
carboxymethyl esters, carbonates, and phosphate  
triesters. Oligonucleotides with these linkages  
can be prepared according to known methods (see,  
20       e.g., Uhlmann et al. (1990) *Chem. Rev.* 90:543-583).

Other modifications include those which are  
internal or at the end(s) of the oligonucleotide  
molecule and include additions to the molecule of  
25       the internucleoside phosphate linkages, such as  
cholesteryl or diamine compounds with varying  
numbers of carbon residues between the amino  
groups and terminal ribose, deoxyribose and  
phosphate modifications which cleave, or crosslink  
30       to the opposite chains or to associated enzymes or  
other proteins which bind to the viral genome.  
Examples of such modified oligonucleotides include  
oligonucleotides with a modified base and/or sugar  
such as arabinose instead of ribose, or a 3', 5'-



-15-

substituted oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position). Other modified oligonucleotides are capped with a nuclease resistance-conferring bulky substituent at their 3' and/or 5' end(s), or have a substitution in one nonbridging oxygen per nucleotide. Such modifications can be at some or all of the internucleoside linkages, as well as at either or both ends of the oligonucleotide and/or in the interior of the molecule.

The preparation of these unmodified and modified oligonucleotides is well known in the art (reviewed in Agrawal et al. (1992) *Trends Biotechnol.* 10:152-158). For example, nucleotides can be covalently linked using art-recognized techniques such as phosphoramidate, H-phosphonate chemistry, or methylphosphoramidate chemistry (see, e.g., Uhlmann et al. (1990) *Chem. Rev.* 90:543-584; Agrawal et al. (1987) *Tetrahedron. Lett.* 28:(31):3539-3542); Caruthers et al. (1987) *Meth. Enzymol.* 154:287-313; U.S. Patent 5,149,798). Oligomeric phosphorothioate analogs can be prepared using methods well known in the field such as methoxyphosphoramidite (see, e.g., Agrawal et al. (1988) *Proc. Natl. Acad. Sci. (USA)* 85:7079-7083) or H-phosphonate (see, e.g., Froehler (1986) *Tetrahedron Lett.* 27:5575-5578) chemistry. The synthetic methods described in Bergot et al. (*J. Chromatog.* (1992) 559:35-42) can also be used.

-16-

A demonstration of the antiviral effect of individual oligonucleotides and methods of the invention is described below.

5           When EBV infects B lymphocytes, the cells become immortalized due to the activation of the latent cycle. Some of the immortalized B cells can become malignant, which is thought to lead to such malignancies as Burkitt's Lymphoma. One such  
10 immortalized cell line is the Akata cell line which is a Type I Burkitt's Lymphoma cell line. It has been discovered that anti-IgG crosslinking of such lymphoma, as well as other specific interventions, triggers the transition of the  
15 latent phase to the lytic cycle by inducing/activating BZLF1. Thus, these cells are useful for testing the ability of various oligonucleotides to inhibit the onset of the lytic cycle and EBV productive replication.

20           In order to determine if antisense oligonucleotides to BZLF1 inhibits productive EBV replication, such a lymphoma cell line was treated with four different concentrations (4, 20, 100 and  
25 200  $\mu$ g/ml) of unmodified or modified oligonucleotide complementary to BZLF1 mRNA, or with a modified or unmodified control oligonucleotide and then with anti-IgG immunoglobulin to induce the lytic cycle (Takada  
30 (1984) *Int. J. Cancer* 33:27-32; Tovey et al. (1978) *Nature* 276:270-272). Inhibition of EBV replication was determined by measuring the activation of the EA<sub>p</sub> gene by counting the percentage of EA<sub>p</sub>-positive cells under fluorescent light after

-17-

staining the cells with human serum, counter-stained with fluorescein labelled goat-anti-human serum.

5           Representative results for EA<sub>p</sub> induction after treatment with 100 µg/ml oligonucleotide are shown in the bar graph in FIG. 1. The results for EA<sub>p</sub> induction after treatment with different concentrations of oligonucleotide are shown as the  
10           dose-response curves in FIGS. 2A and 2B. These results demonstrate that both modified and unmodified oligonucleotides complementary to portions of the BZLF1 RNA (the "antisense" oligonucleotide) inhibit EA<sub>p</sub> induction more  
15           efficiently than do the modified and unmodified control oligonucleotides. Furthermore, modified antisense oligonucleotides at 20 µg/ml has about the same effect as unmodified antisense oligonucleotides at 200 µg/ml, probably due to the  
20           short halflife of the unmodified oligonucleotides (having phosphodiester internucleotide linkages) in the cell.

          Another way to measure the ability of  
25           oligonucleotides of the invention to inhibit EBV replication is to measure EBV DNA synthesis and to determine the conformation that the newly synthesized DNA assumes. During latency, the DNA is circular; during lytic infection, the DNA  
30           assumes a linear conformation. Measurement of DNA synthesis and conformation can be accomplished as follows. Briefly, 10<sup>6</sup> µg/ml cells treated with no oligonucleotides (untreated) or with four different concentrations of either an unmodified

-18-

or modified control or antisense oligonucleotide (as described above), or 5 ml of the cell supernatants from each untreated or treated cell batch, were loaded into the wells of a Gardella gel (5 *J. Virol.* (1984) 50:248-254) as described in the examples below. The supernatants are monitored to detect virus release from the cells. The gels after transfer and hybridization with a  $^{32}\text{P}$ -labelled EBV probe were counted for  $^{32}\text{P}$  in the 10 linear and circular EBV DNA range, and subjected to autoradiography. The cpm of the  $^{32}\text{P}$  are shown in TABLE 1. This Table also lists the ratio of counts in the linear EBV DNA range in the gels, compared to their respective untreated (control) 15 cells and also after partially correcting the control results for the variations seen.

-19-

TABLE I

	4 $\mu$ g/ml		20 $\mu$ g/ml		100 $\mu$ g/ml		200 $\mu$ g/ml	
	CPM	Ratio	CPM	Ratio	CPM	Ratio	CPM	Ratio
No antisense	39	1.00	75	1.00	146	1.00	88	1.00
modified antisense	34	0.86	20	0.27	33	0.23	24	0.26
modified control	43	1.09	57	0.76	149	1.01	60	0.69
unmodified antisense	47	1.20	82	1.09	116	0.79	32	0.37
unmodified control	64	1.60	78	1.04	164	1.12	117	1.3

-20-

These results show that EBV DNA replication is reduced by 73% when infected cells are treated with 20  $\mu\text{g/ml}$  modified antisense oligonucleotides, with little variation at higher concentrations, and is reduced by 63% when 200  $\mu\text{g/ml}$  unmodified antisense oligonucleotide was used. In contrast, modified and unmodified unrelated (control) oligonucleotides have little or no inhibitory effect on replication.

Yet another way to measure the ability of the antisense oligonucleotides of the invention to inhibit EBV replication is to measure the presence of the late gene product viral capsid antigen (VCA), the major capsid protein. Using the same Akata cells treated as described above, slides were made, frozen, and stained for VCA using fluorescently labelled antibody to VCA. The percentage of VCA-positive cells are then counted and plotted versus the oligonucleotide concentration used.

The results shown graphically in the dose response curves in FIGS. 3A and 3B are quantitatively similar to the EA<sub>p</sub> staining shown in FIGS. 2A and 2B: 32% of the induced cells are VCA-positive when untreated; when treated with modified antisense oligonucleotide, the percentage of VCA-positive cells is reduced in a dose-dependent fashion to 15%, 12%, and 10% at 20  $\mu\text{g/ml}$ , 100  $\mu\text{g/ml}$ , and 200  $\mu\text{g/ml}$ , respectively. Similarly, treatment with unmodified antisense oligonucleotides reduces the percentage VCA-positive cells to 22% and 16% at 100  $\mu\text{g/ml}$  and 200

-21-

$\mu\text{g/ml}$ , respectively, while treatment with control oligonucleotides has less of an inhibitory effect than either modified or unmodified antisense oligonucleotide.

5

It is known that BZLF1 mRNA can be found in the nucleus of Akata cells 20 minutes after induction by IgG (Mellinghoff et al. (1991) Virology 185:922-928). Thus, to determine to  
10 determine the most effective time to treat the cells with the antisense oligonucleotide of the invention, untreated Akata cells or Akata cells treated with various control or antisense oligonucleotides at different times before  
15 induction with IgG were subjected to gel analysis to determine the extent of DNA synthesis as described above.

The results are shown in FIG. 4. The numbers  
20 under each lane are the fractional amounts of counts in the linear (L) EBV DNA range compared to the counts detected in untreated, induced cells (lane 1). These results show that the addition of antisense oligonucleotide to BZLF1 mRNA 3 hours  
25 before IgG activation of the lytic cycle reduces viral DNA synthesis over two fold in comparison with the addition of oligonucleotide added at the same time or three hours after IgG activation. Thus, the most effective time to treat latently  
30 effected cells is before induction.

Like the Akata cells mentioned above, P3HR-1 cells are also useful for testing the ability of various oligonucleotides to inhibit lytic

-22-

replication. P3HR-1 is another cell line derived from Burkitt's Lymphoma. This line produces EBV even when uninduced. Furthermore, because the virus in these cells is temperature sensitive, infected cells produce more virus at 33°C than at 37°C.

When uninduced P3HR-1 cells are treated with modified antisense oligonucleotides at 100 µg/ml or 200 µg/ml at day 0, subcultured every 2 days with medium containing 100 µg/ml or 200 µg/ml oligonucleotide, respectively at 33°C, and the amounts of linear (L) and circular (C) EBV DNA measured by detecting the counts of <sup>32</sup>P in Gardella gels as described above, EBV DNA synthesis is shown to be inhibited. The results are shown in the autoradiograms in FIGS. 5A and 5B. The numbers under each lane are the fractional amount of counts in the linear (L) EBV DNA range compared to the counts in cells not treated with antisense oligonucleotide. FIG. 5B demonstrates that 200 µg/ml modified antisense oligonucleotide reduces the amounts of EBV DNA synthesis 52%, 68%, and 79% at days 6, 10 and 16, respectively, in contrast to the control modified oligonucleotides which reduce DNA synthesis to a lesser extent.

Thus, the modified and unmodified oligonucleotides of the invention have been demonstrated to have the ability to inhibit the onset of EBV lytic replication in latently infected cells, and to inhibit further EBV replication in cells already producing virus.



-23-

Therefore, these oligonucleotides are useful for preventing lytic replication by EBV in latently infected cell cultures, thereby keeping the cell culture in a latently infected state.

5

That the oligonucleotides of the invention are not toxic to the cells was determined by comparing the rate of <sup>3</sup>H-thymidine incorporation into the newly synthesized DNA of oligonucleotide-treated cells and untreated cells. There is no difference in the rate of DNA synthesis, indicating the relative health of the cells.

10

The present invention further provides a therapeutic composition having antiviral activity against EBV lytic infection. The composition includes at least an oligonucleotide of the present invention, along with a physiologically acceptable carrier.

15

20

As used herein, a "physiologically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

25

30

-24-

This therapeutic formulation may be used to inhibit, reduce or prevent the replication of EBV in cells through the activity of the oligonucleotides in the composition. EBV-infected cell are then treated with the therapeutic formulation in an amount sufficient to enable the binding of the oligonucleotide to the RNA in the infected cells. In this way, the binding of the oligonucleotide to the EBV RNA inhibits the expression and replication of the virus.

The oligonucleotides of the invention may also be used to treat EBV infection in humans. In this method, the composition is administered once in a therapeutically effective amount or repeatedly in less than therapeutic amounts. Administration may be by intravenous or intraperitoneal injection, or by intranasal, oral, transdermal, or subcutaneous administration. Effective dosages of the oligonucleotide and modes of its administration in the treatment of EBV can be determined by routine experimentation. The pharmaceutical forms suitable for injectable or other use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile. It must be stable under the conditions of manufacture and storage and may be preserved against the contaminating action of microorganisms, such as bacterial and fungi. The carrier can be a solvent or dispersion medium. The prevention of the action of microorganisms can be brought about by various antibacterial and

-25-

antifungal agents. Prolonged absorption of the injectable therapeutic agents can be brought about by the use of the compositions of agents delaying absorption.

5

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

10

### EXAMPLES

#### 1. Cell Lines

15

Akata (Takada et al. (1989) *J. Virol.* 63:445-449), an EBV-nonproducer Burkitt's lymphoma cell line, and P3HR-1 (Hinuma et al. (1967) *J. Virol.* 1:1045-1051), an EBV-producer cell line, were maintained in RPMI 1640 medium supplemented with penicilline (100 IU/ml), streptomycin (100 µg/ml), and 10% heat-inactivated fetal calf serum in a humidified atmosphere of 5% CO<sub>2</sub> in air. Akata and P3HR-1 cells were passaged at 37°C and 33°C, respectively. The production of P3HR-1 cells is higher upon incubation at 33°C than at 37°C.

25

#### 2. Oligonucleotides

30

Unmodified (phosphodiester-linked (PO)) and modified (phosphorothioate-linked) (PS) oligonucleotides were synthesized by H-phosphonate chemistry using on an automated DNA synthesizer. These oligonucleotides were purified as described

-26-

in U.S. patent application Serial No. 07/311,111 ("METHOD OF SEPARATING OLIGONUCLEOTIDES FROM A MIXTURE," by Agrawal and Zamecnik, filed February 15, 1989, and recently allowed). A 25-mer  
5 oligonucleotide having the sequence 5'-TTT GGG TCC ATC ATC TTC AGC AAA G-3' (SEQ ID NO:1), which is the flanking sequence of the translational initiation codon (AUG) of BZLF1 transcript, was used as a BZLF1 antisense oligonucleotide. Two  
10 other BZLF1 antisense oligonucleotides which are immediately adjacent to each other and partially overlap with the SEQ ID NO:1 antisense oligonucleotide were also tested: the 20mer 5'-CAT CAT CTT CAG CAA AGA TA-3' (SEQ ID NO:2) and  
15 the 20mer 5'-TCA GAA GTC GAG TTT GGG TC-3' (SEQ ID NO:3). SEQ ID NO:2 antisense oligonucleotide is complementary to the sequence starting with the 5' untranslated region and ending with the two copies of AUG with which the open reading frame starts.  
20 SEQ ID NO:3 antisense oligonucleotide hybridizes to a region immediately downstream of the translational initiation codon of the mRNA derived from the BZLF1 gene. 25mer modified (phosphorothioate-linked) and PO-linked  
25 oligonucleotides with unrelated random sequences were employed as controls.

### 3. Oligonucleotide Treatment of Cells

30 Logarithmically growing Akata cells were resuspended to a final concentration of  $1 \times 10^6$  cells/ml with fresh medium. The cells were incubated in the presence of various concentration of modified or PO-linked antisense of control

-27-

oligonucleotides for 3 hours at 37°C in 5% CO<sub>2</sub>, and then stimulated with anti-human IgG antibodies (Cappel, West Chester, PA) at a concentration of 100 µg/ml for 24 hours.

5

For the treatment of P3HR-1 cells with modified oligonucleotides, the cells (1 x 10<sup>6</sup> cells/ml) received the oligonucleotides at the indicated concentrations, and the incubations were continued for 2 days at 33°C in 5% CO<sub>2</sub>. Every other day cell number was counted, and aliquots were taken for assay by EBV DNA analysis and immunofluorescence. After adjustment of the cell concentration to 1 x 10<sup>6</sup> cells/ml by adding fresh medium, the cells were treated again with modified oligonucleotides. Up to 9 treatments over a period of 18 days were performed.

10

15

#### 4. EBV DNA Analysis

20

The form and quantity of EBV DNA were determined by gel electrophoresis and genomic hybridization of the blotted electrophoretic patterns (Gardella et al. (1984) *J. Virol.* 50:248-254). Briefly, 0.5 x 10<sup>6</sup> and 1.0 x 10<sup>6</sup> cells were suspended in 100 µl of sample buffer containing 15% Ficoll and 0.01% bromophenol blue in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) and loaded into the well of a vertical 0.75% agarose gel. Lysis buffer (100 µl), containing 5% Ficoll, 1% sodium dodecyl sulfate (SDS), 1.5 mg of pronase per ml, and 0.05% xylene cyanol green in TBE buffer, was layered over the samples. Electrophoresis was carried out at 20 V

25

30

-28-

for 2 hours and then at 80 V for 16 hours at 4°C. The DNA in the gel was partially depurinated with 0.35 M HCl, denatured with 0.4 M NaOH in 0.5 M NaCl, and transferred to nylon-membrane filters using an alkaline transfer protocol. The papers were baked at 80°C for 1 hour, treated with prehybridization buffer, and hybridized with a random-primed <sup>32</sup>P-labeled EBV *Bam*HI-W probe. After being washed to remove unhybridized probe, the filters were exposed to Kodak XAR films at -80°C with intensifying screens.

#### 5. Immunofluorescence

Indirect immunofluorescence was used for staining of early antigen (EA) and viral capsid antigen (VCA). EA or VCA was detected, respectively, with a serum (anti-EA 1:320; anti-VCA 1:320) from a patient with nasopharyngeal carcinoma, or with a seropositive serum (anti-VCA 1:160; anti-EA less than 1:10) from a healthy donor. Cells were washed with phosphate buffered saline (PBS) and spotted on a glass slide, dried, and fixed in chilly acetone for 15 minutes. The fixed smears were incubated with the serum at 37°C for 40 minutes. After being washed in PBS, the slides were incubated with the FITC-conjugated goat anti-human IgG (Cappel, West Chester, PA) at 37°C for 40 minutes. The slides were washed and mounted in 1:1 glycerol/PBS, and examined under a fluorescence microscope. At least 500 cells were counted for each determination.

-29-

6. Incorporation of  $^3\text{H}$ -Thymidine

5        Either stimulated or unstimulated Akata cells  
( $2 \times 10^6$ ) were incubated with modified or PO-  
linked oligonucleotides for 24 hours. The cells  
were then pulsed with 1  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine  
(NEN/DuPont) for 4 hours at  $37^\circ\text{C}$ . After brief  
cell lysis with 0.4 M NaOH, the lysates were  
10       placed on fiberglass filtermats and washed  
extensively. The dried filters were transferred  
to scintillation vials containing 3 ml of  
scintillant and counted.

EQUIVALENTS

15

Those skilled in the art will recognize, or  
be able to ascertain, using no more than routine  
experimentation, numerous equivalents to the  
specific substances and procedures described  
20       herein. Such equivalents are considered to be  
within the scope of this invention, and are  
covered by the following claims.

-30-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Mulder, Carel
- (ii) TITLE OF INVENTION: Oligonucleotides  
With Anti-Epstein-  
Barr Virus Activity
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Lappin & Kusmer
  - (B) STREET: 200 State Street
  - (C) CITY: Boston
  - (D) STATE: Massachusetts
  - (E) COUNTRY: USA
  - (F) ZIP: 02109
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Kerner, Ann-Louise
  - (B) REGISTRATION NUMBER: 33,523
  - (C) REFERENCE/DOCKET NUMBER: HYZ-017PCT
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 617-330-1300
  - (B) TELEFAX: 617-330-1311

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear



-31-

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: YES  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
TTTGGGTCCA TCATCTTCAG CAAAG 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 20 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: YES  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:  
CATCATCTTC AGCAAAGATA 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 20 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: YES  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:  
TCAGAAGTCG AGTTTGGGTC 20

-32-

What is claimed is:

1. A synthetic oligonucleotide complementary to  
and hybridizable with a portion of the BZLF1 RNA  
5 of Epstein-Barr virus.
2. The oligonucleotide of claim 1 which includes  
at least 12 nucleotides.
- 10 3. The oligonucleotide of claim 1 which includes  
from 12 to 60 nucleotides.
4. The oligonucleotide of claim 1 which is  
modified.
- 15 5. The oligonucleotide of claim 2 which is  
modified.
6. The oligonucleotide of claim 3 which is  
20 modified.
7. The oligonucleotide of claim 4 comprising at  
least one internucleotide linkage selected from  
the group consisting of alkylphosphonate,  
25 phosphorothioate, phosphorodithioate,  
alkylphosphonothioate, phosphoramidate, phosphate  
ester, carbamate, carbonate, phosphate triester,  
acetamidate, carboxymethyl ester, and combinations  
thereof.

30

-33-

8. The oligonucleotide of claim 5 comprising at least one internucleotide linkage selected from the group consisting of alkylphosphonate, phosphorothioate, phosphorodithioate, alkylphosphonothioate, phosphoramidate, phosphate ester, carbamate, carbonate, phosphate triester, acetamdate, carboxymethyl ester, and combinations thereof.
9. The oligonucleotide of claim 6 comprising at least one internucleotide linkage selected from the group consisting of alkylphosphonate, phosphorothioate, phosphorodithioate, alkylphosphonothioate, phosphoramidate, phosphate ester, carbamate, carbonate, phosphate triester, acetamdate, carboxymethyl ester, and combinations thereof.
10. The oligonucleotide of claim 7 comprising at least one phosphorothioate internucleotide linkage.
11. The oligonucleotide of claim 8 comprising at least one phosphorothioate internucleotide linkage.
12. The oligonucleotide of claim 9 comprising at least one phosphorothioate internucleotide linkage.
13. The oligonucleotide of claim 1 which comprises at least one deoxyribonucleotide.

-34-

14. The oligonucleotide of claim 2 which comprises at least one deoxyribonucleotide.

5 15. The oligonucleotide of claim 3 which comprises at least one deoxyribonucleotide.

16. The oligonucleotide of claim 1 which comprises at least one ribonucleotide.

10 17. The oligonucleotide of claim 2 which comprises at least one ribonucleotide.

15 18. The oligonucleotide of claim 3 which comprises at least one ribonucleotide.

19. The oligonucleotide of claim 1 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

20 20. The oligonucleotide of claim 2 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

25 21. The oligonucleotide of claim 3 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

30 22. A synthetic oligonucleotide which has antiviral activity against Epstein-Barr virus effected by hybridization with a portion of the viral BZLF1 RNA.

-35-

23. A pharmaceutical composition comprising the oligonucleotide of claim 1 and a pharmaceutically acceptable carrier.
- 5 24. A pharmaceutical composition comprising the oligonucleotide of claim 2 and a pharmaceutically acceptable carrier.
- 10 25. A pharmaceutical composition comprising the oligonucleotide of claim 3 and a pharmaceutically acceptable carrier.
- 15 26. A pharmaceutical composition comprising the oligonucleotide of claim 4 and a pharmaceutically acceptable carrier.
- 20 27. A pharmaceutical composition comprising the oligonucleotide of claim 5 and a pharmaceutically acceptable carrier.
28. A pharmaceutical composition comprising the oligonucleotide of claim 6 and a pharmaceutically acceptable carrier.
- 25 29. The pharmaceutical composition comprising the oligonucleotide of claim 19.
- 30 30. The pharmaceutical composition comprising the oligonucleotide of claim 20.
31. The pharmaceutical composition comprising the oligonucleotide of claim 21.

-36-

32. A method of inhibiting Epstein-Barr virus replication in a cell comprising the step of administering to the cell a therapeutic amount of the pharmaceutical composition of claim 23.

5

33. A method of inhibiting Epstein-Barr virus replication in a cell comprising the step of administering to the cell a therapeutic amount of the pharmaceutical composition of claim 24.

10

34. A method of inhibiting Epstein-Barr virus replication in a cell comprising the step of administering to the cell a therapeutic amount of the pharmaceutical composition of claim 25.

15

35. A method of treating Epstein-Barr virus infection comprising the step of administering to an infected cell the pharmaceutical composition of claim 23.

20

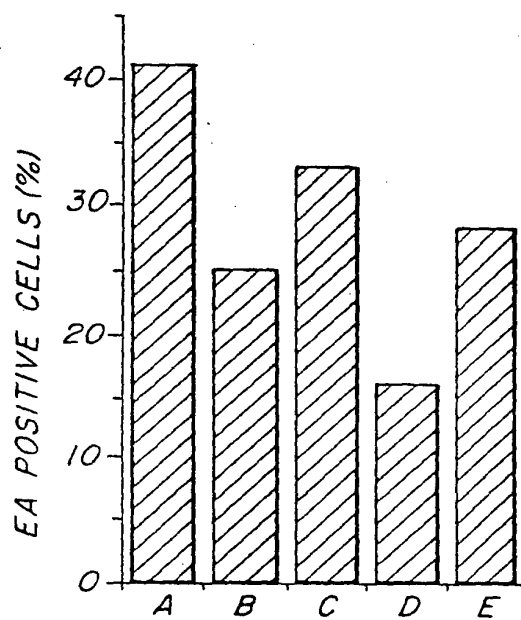
36. A method of treating Epstein-Barr virus infection comprising the step of administering to an infected cell the pharmaceutical composition of claim 24.

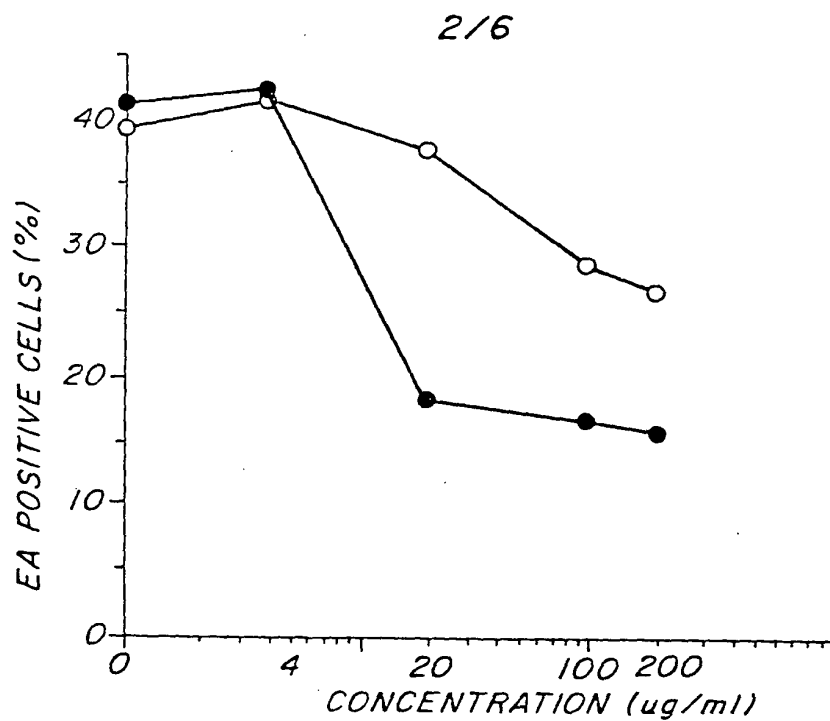
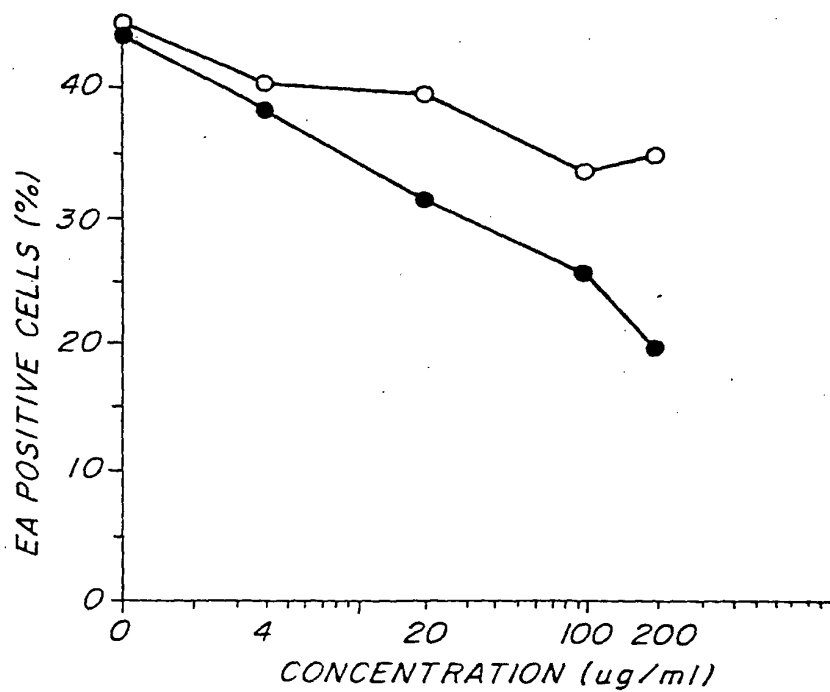
25

37. A method of treating Epstein-Barr virus infection comprising the step of administering to an infected cell the pharmaceutical composition of claim 25.

30

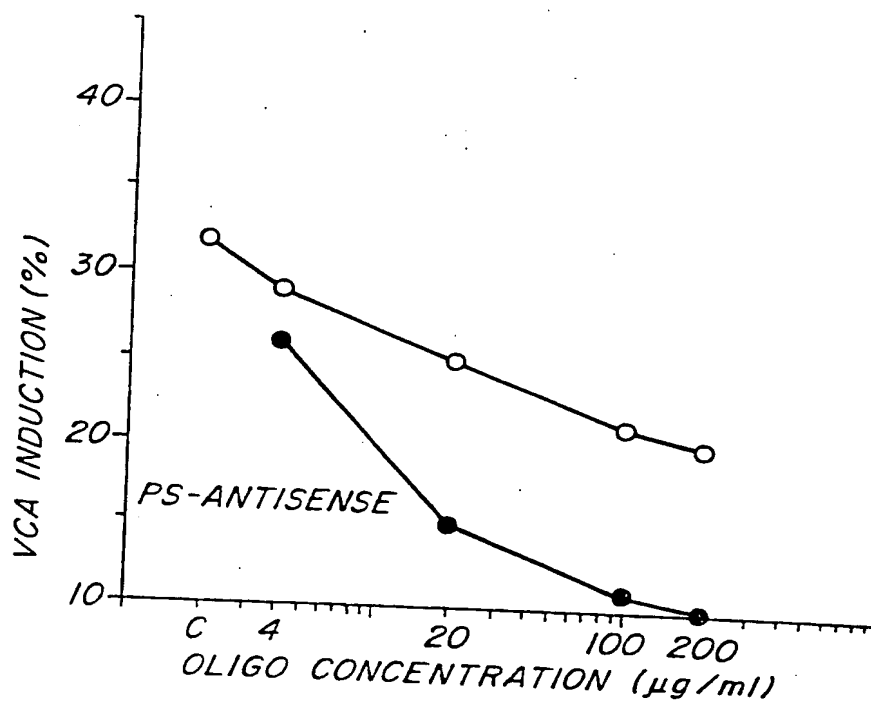
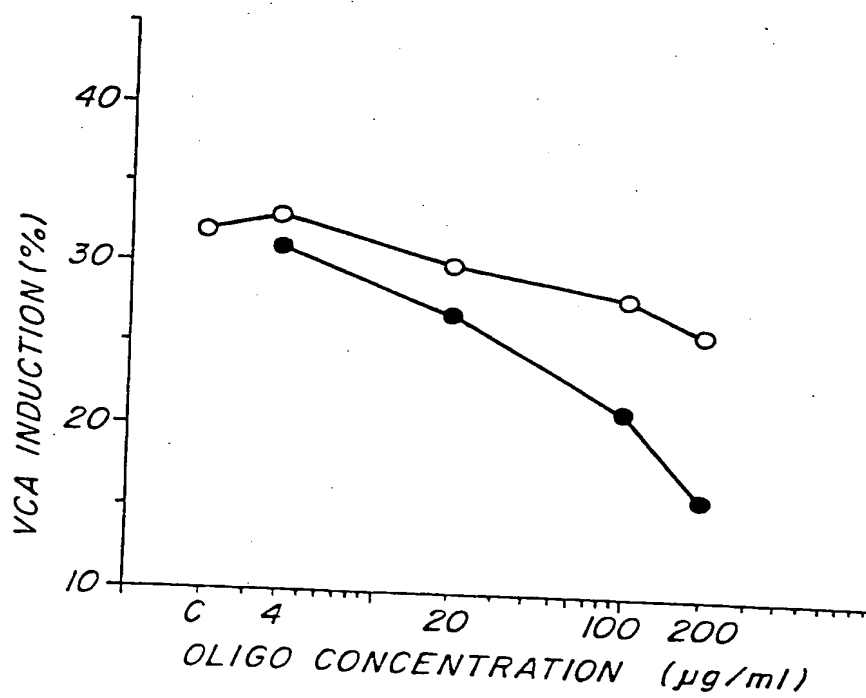
1/6

*FIG. 1*

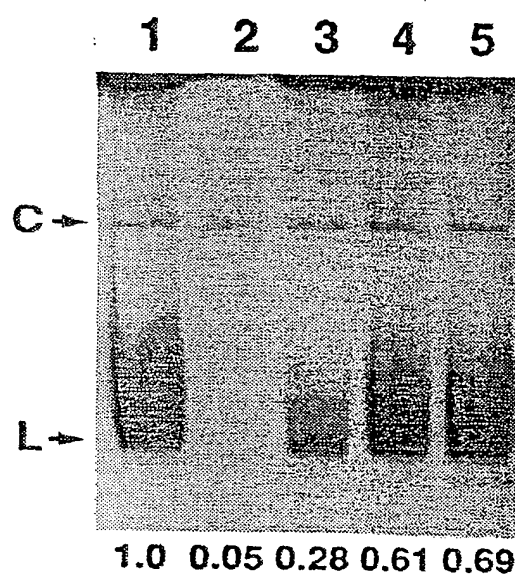
*FIG. 2A**FIG. 2B*



3/6

**FIG. 3A****FIG. 3B**

4/6

*FIG. 4*

5/6

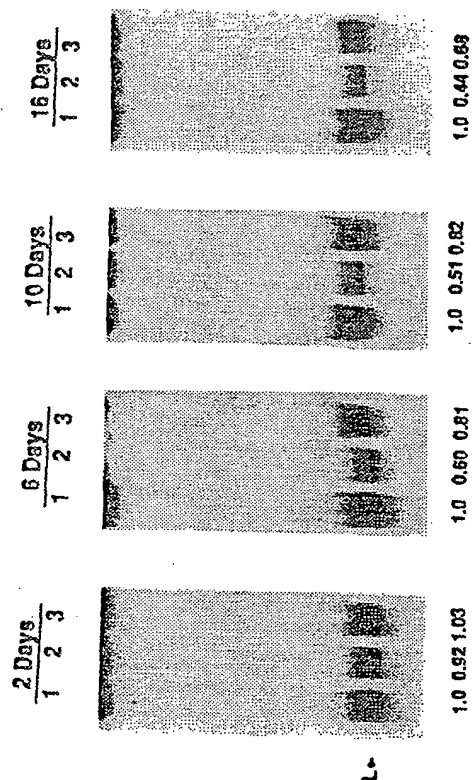


FIG. 5A

6/6

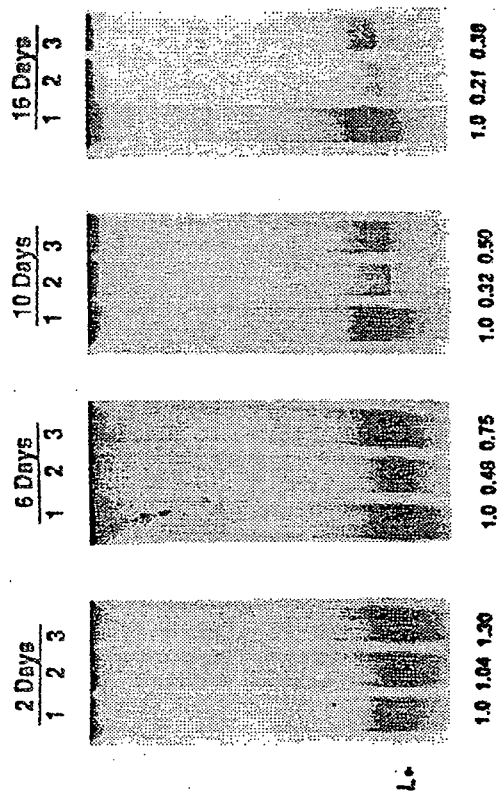


FIG. 5B

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/02082

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07H21/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 93 07882 (ISI PHARMACEUTICALS INC) 29 April 1993	1-37
Y	see page 17, line 17 - line 30 ---	1-21
Y	JOURNAL OF VIROLOGY, vol. 61, no. 10, October 1987 WASHINGTON DC (US), pages 3120-3132, BIGGIN M. ET AL 'Epstein-Barr Virus Gene Expression in P3HR1-Superinfected Raji Cells' see figure 6 -----	1-21

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

29 June 1995

Date of mailing of the international search report

06.07.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Day, G

## INTERNATIONAL SEARCH REPORT

### Box I Observations where certain claims were found unsearchable (Continuation)

This international search report has not been established in respect of certain claims under Article 17 of the Patent Cooperation Treaty (PCT).

1. ☒ Claims Nos.: 32-37  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark - Although claims 32-37 are directed to a method of treatment of the human/animal body (Art.52(4) EPC) the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

information on patent family members

PCT/US 95/02082

Form PCT/ISA/210 (patent family annex) (July 1992)